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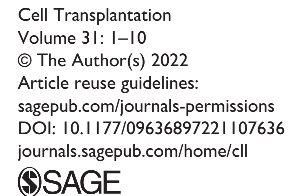
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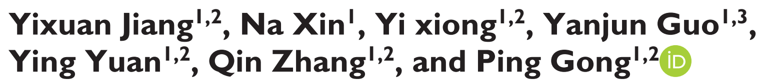
αCGRP Regulates Osteogenic Differentiation of Bone Marrow Mesenchymal Stem Cells Through ERK1/2 and p38 MAPK Signaling Pathways


Yixuan Jiang[1,2], Na Xin[1], Yi xiong[1,2], Yanjun Guo[1,3], Ying Yuan[1,2], Qin Zhang[1,2], and Ping Gong[1,2]

Abstract

As a typical neuropeptide richly distributed in central and peripheral nervous systems, α-calcitonin-gene-related peptide (αCGRP) has recently been found to play a crucial role in bone development and metabolism, but the mechanisms involved are not fully uncovered. Here, this study aimed to investigate the effects and underlying molecular mechanisms of αCGRP in regulating the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs). Using microarray technology, gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) analyses revealed that osteogenic properties of BMSCs were facilitated and mitogen-activated protein kinase (MAPK) signaling pathway was upregulated by αCGRP in this process. Through western blot assay, we proved that αCGRP led to an increased phosphorylation level of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and p38 MAPK signaling cascades in a time-dependent manner. And αCGRP could promote differentiative capacity of BMSCs, showing upregulated mRNA and protein expression level of alkaline phosphatase (Alp), collagen type 1 (Col-1), osteopontin (Opn), and runt-related transcription factor 2 (Runx2), as well as increased ALP activity and calcified nodules. The addition of ERK1/2 or p38 MAPK inhibitor—U0126 or SB203580, resulted in an impaired osteogenic differentiation of BMSCs. Besides, inactivation of this signal transduction had negative impacts on proliferative activity and apoptotic process of αCGRP-mediated BMSCs. Our findings demonstrated that MAPK signaling pathway, at least in part, was responsible for the enhanced BMSCs' osteogenesis induced by αCGRP, which might offer us promising strategies for bone-related disorders.

Keywords

α-calcitonin gene related peptide (αCGRP), bone marrow mesenchymal stem cells (BMSCs), mitogen-activated protein kinase (MAPK), osteogenic differentiation, osteogenesis

Introduction

Bone-related disorders, including osteoporosis, fracture, and so on, are long-standing problems posing challenges to human health, with impaired life quality for millions of individuals worldwide[1]. Whether we can improve the osteogenesis process in the dynamic remodeling of bone has become the hot topic of discussion in this realm. Bone tissue is composed of various cells, of which bone marrow mesenchymal stem cells (BMSCs) exert indispensable functions. This stem cell population is the most frequently used cell type for skeletal tissue engineering applications, since it has the potential of self-renewal and osteoblastic differentiation[2,3]. The secretion of paracrine factors by BMSCs is also an effective approach to improve tissue repair and regeneration[4]. Additionally, it is described that promoting the proliferation and migration of BMSCs is necessary for bone healing, which highlights the significance of BMSCs in maintaining bone homeostasis and supporting skeletal integrity[5,6].

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Over the decades, most research focuses attention on strategies which could accelerate bone repair through facilitating direct osteogenesis. Since natural bone is a highly mineralized and dynamic living tissue, it should be noted that the bone system consists of not only bone tissue, but also nerves and vessels, with intricate microenvironment[3,7]. Emerging evidence has tested out the great importance of neural involvement in regulating bone formation, repair, and regeneration[8–10]. At the site with high osteogenic activity, nerve fibers accompanied rich capillary network were observed in the developing skeleton[11]. While deletion or reduction of sensory innervation could damage the skeletal integrity and block the process of fracture healing[12]. Moreover, some neurotransmitters are implicated in skeletal development and homeostasis[9], offering a novel insight for further investigations.

Composed of 37-amino acid, calcitonin-gene-related peptide (CGRP) has become a hot topic in this field. It is a kind of typical neuropeptide richly distributed in central and peripheral nervous systems, participating in angiogenesis, algesia, and so forth[13,14]. There are two isoforms of CGRP, namely αCGRP and βCGRP. In general, αCGRP is regarded as an attractive candidate to orchestra bone metabolism, while βCGRP is rarely involved in bone remodeling[15,16]. The presence of αCGRP receptors on the surface membrane of BMSCs and other bone-related cells provides us the further evidence of its modulatory role in osteogenesis[17,18]. A previous experiment has indicated that αCGRP might play a critical role in the regulation of the age-related switch between osteogenesis and adipogenesis in BMSCs[19]. *In vivo*, αCGRP is suggested to be an osteoanabolic mediator in fracture healing in long bones[20]. Our previous research has also reported that the absence of αCGRP could impede peri-implant osseointegration in mouse femurs, indicating its important influences on local bone metabolism[21]. However, there is still a lack of evidence revealing the molecular mechanisms involved, which makes it more difficult to understand the precise role of αCGRP in osteogenesis.

Based on these, the core issue of this study was to investigate biological functions of αCGRP in BMSCs and the intrinsic mechanisms involved. This work would provide us novel perspectives of the osteogenic ability of αCGRP, which might offer new strategies for the treatment of bone-related disorders.

Materials and Methods

Culture and Identification of BMSCs

This study was approved by the Ethics Committee of West China Hospital of Stomatology, Sichuan University (No. WCHSIRB-D-2017-162). Cells were harvested from medullary cavities of femur and tibia of C57BL/6 mice (2–3 weeks old) and moved into culture flasks containing α-MEM (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin sulfate, which were placed in an incubator at 37°C with 5% CO_2. Fresh medium was replaced every 2 days and cells in passage 2–3 were used for the following experiments.

BMSCs were identified using alizarin red staining and Oil Red O staining. First, cells were cultured in osteogenic induction medium (α-MEM, 10% FBS, 50 μg/ml ascorbic acids, antibiotics, 10 mM β-glycerophosphate, and 100^{-8} M dexamethasone [Gibco, USA]) for 21 days. Afterward, cells were fixed by 4% paraformaldehyde for 30 min and stained by alizarin red (Sigma, USA) following manufacturer's instructions. Second, cells were cultured in the adipogenic differentiation medium (α-MEM, 10% FBS, 1 μmol/l dexamethasone, 0.05 mmol/l 3-isobutyl-1-methylxanthine, 10 μmol/ml bovine insulin and 200 μmol/l indomethacin) for 7 days. Subsequently, cells were fixed and stained with Oil Red O (Sigma, USA) and then rinsed with 60% isopropanol. Lipid droplets were explored by phase-contrast microscopy.

Microarray Analysis

To explore the effect of αCGRP on BMSCs, cells were seeded at a density of 2×10^5 cells per well in 6-well plates and allocated to two groups: (1) αCGRP overexpression group: BMSCs transfected with αCGRP overexpression lentiviral vector and (2) empty vector control group: BMSCs transfected with empty lentiviral vector. Lentivirus-CGRP vector and empty vector used in this study were obtained from GENECHEM (GENECHEM Co., Shanghai, China). For these two groups, the lentivirus was used with Enhancer reagent and 5 μg/ml polybrene (GENECHEM Co., Shanghai, China). BMSCs were incubated with lentiviral vector at an MOI of 40 for 8 h. Then fresh α-MEM medium supplied with 10% FBS was replaced. Cells were collected and microarray gene expression analysis was performed on Mouse lncRNA Microarray V3 (4×180 K, Design ID:084388, Agilent Technologies, USA). In brief, total RNA from different groups was extracted, amplified, labeled, and hybridized onto the microarray chip according to manufacturer's instructions. After washing, the arrays were scanned using Agilent Scanner G2505C (Agilent Technologies, USA). Then images were analyzed by Feature Extraction software (version10.7.1.1, Agilent Technologies, USA). Genespring (version 13.1, Agilent Technologies, USA) was employed to process the data. Hierarchical Clustering was conducted to display the distinguishable genes' expression pattern among samples. In addition, gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) analysis were applied to investigate the roles of these genes.

Cell Treatment

To further elucidate the role of mitogen-activated protein kinase (MAPK) signal in αCGRP-mediated BMSCs, cells were divided into following groups: (1) control group, (2) αCGRP group: αCGRP (10^{-8}M, Phoenix, USA) was

Table 1. Primer Sequences for Real Time-qPCR.

Gene	Forward primer 5′–3′	Reverse primer 5′–3′
Alp	AACCCAGACACAAGCATTCC	GCCTTTGAGGTTTTTGGTCA
Col-1	GAGCGGAGAGTACTGGATCG	GCTTCTTTTCCTTGGGGTTC
Opn	CCCGGTGAAAGTGACTGATT	TTCTTCAGAGGACACAGCATTC
Runx2	CCAACCGAGTCATTTAAGGCT	GCTCACGTCGCTCATCTTG
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

Alp: alkaline phosphatase; Col-1: collagen type 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Opn: osteopontin; qPCR: quantitative polymerase chain reaction; Runx2: runt-related transcription factor 2.

added to BMSCs, (3) αCGRP-U0126 group: BMSCs were preincubated with 10 μM U0126 (extracellular signal-regulated kinases 1 and 2 (ERK1/2) pathway inhibitor, Cell signaling technology, USA) and αCGRP, and (4) αCGRP-SB203580 group: BMSCs were preincubated with 10μM SB203580 (p38 MAPK pathway inhibitor, Cell signaling technology, USA) and αCGRP.

Western Blot Analysis

Cells were subjected to lysis buffer (Keygen total protein extraction kit, Keygen Biotech, China). Protein extracts were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene difluoride membranes (Millipore Corp., USA). Subsequently, the membranes were probed overnight with primary antibodies at 4°C. The next day, they were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000). Then the immunoreactive bands were exposed and immunoblot images were obtained by Quantity One (Bio-Rad, USA). The primary antibodies used in this study were as follows: p-ERK1/2 (1:600, Santa Cruz), p-p38 (1:600, Santa Cruz), alkaline phosphatase (Alp, 1:500, Abcam), collagen type 1 (Col-1, 1:500, Abcam), runt-related transcription factor 2 (Runx2, 1:500, Abcam), and osteopontin (Opn, 1:500, Abcam). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000, SAB) was applied as internal control.

Cell Counting Kit-8 (CCK-8) and Caspase-3 Assay

For cell viability, BMSCs were seeded at a density of 5×10^3/well in 96-well plates and detected at different time points using CCK-8 assay (Dojindo, Japan). The optical density (OD) value was determined at 450 nm by a microplate reader (NanoDrop, USA). Apoptosis assay was implemented after 7-d culture in different groups. Caspase-3 activity was examined by quantifying the degradation of the fluorometric substrate DEVD (Biomol Research Labs).

RNA Isolation and Real-Time qPCR

Cells were harvested and cellular RNAs were extracted by Trizol Reagent (Invitrogen, USA). Using Prime Script Reverse Transcriptase (Takara, Japan), reverse transcriptions were performed and cDNA was synthesized. After that, cDNA was amplified with SYBR Premix Ex Taq (Takara, Japan) and real-time qPCR was carried out by ABI 7300 real-time PCR system (Applied Biosystems, USA). The mRNA expression levels were tested by normalizing to endogenous housekeeping gene GAPDH. Primer sequences were listed in Table 1.

ALP Activity and Alizarin Red Staining

BMSCs were cultured in osteogenic induction medium as previously described. After 7-day treatment, cells were harvested, fixed by 4% paraformaldehyde for 30 min and stained by ALP staining kit (Beyotime, China). Besides, ALP activity was calculated at 4d and 7d using Alp Assay Kit (Beyotime, China) and total protein concentration were estimated by BCA Protein Assay Kit (Beyotime, China). Then results were normalized to total protein level as nanomoles of produced p-nitrophenol per min per mg of protein (nmol/min/mg protein). After 21-day osteogenic induction, alizarin red staining (Sigma, USA) was conducted according to manufacturer's protocols.

Statistical Analysis

Statistical analysis was performed by SPSS 20.0 software (SPSS, Inc., Chicago, IL). Data are presented as mean ± SD with a minimum of three independent samples. Statistically significant differences were calculated using one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. The value of $P < 0.05$ was considered to be statistically significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Results

αCGRP Affects Gene Expressions and Biological Processes in BMSCs

Primary BMSCs were successfully isolated and cultured as mineralized calcium nodules or lipid droplets were observed (Fig. 1A). To determine the gene expression pattern among samples, hierarchical clustering was constructed.

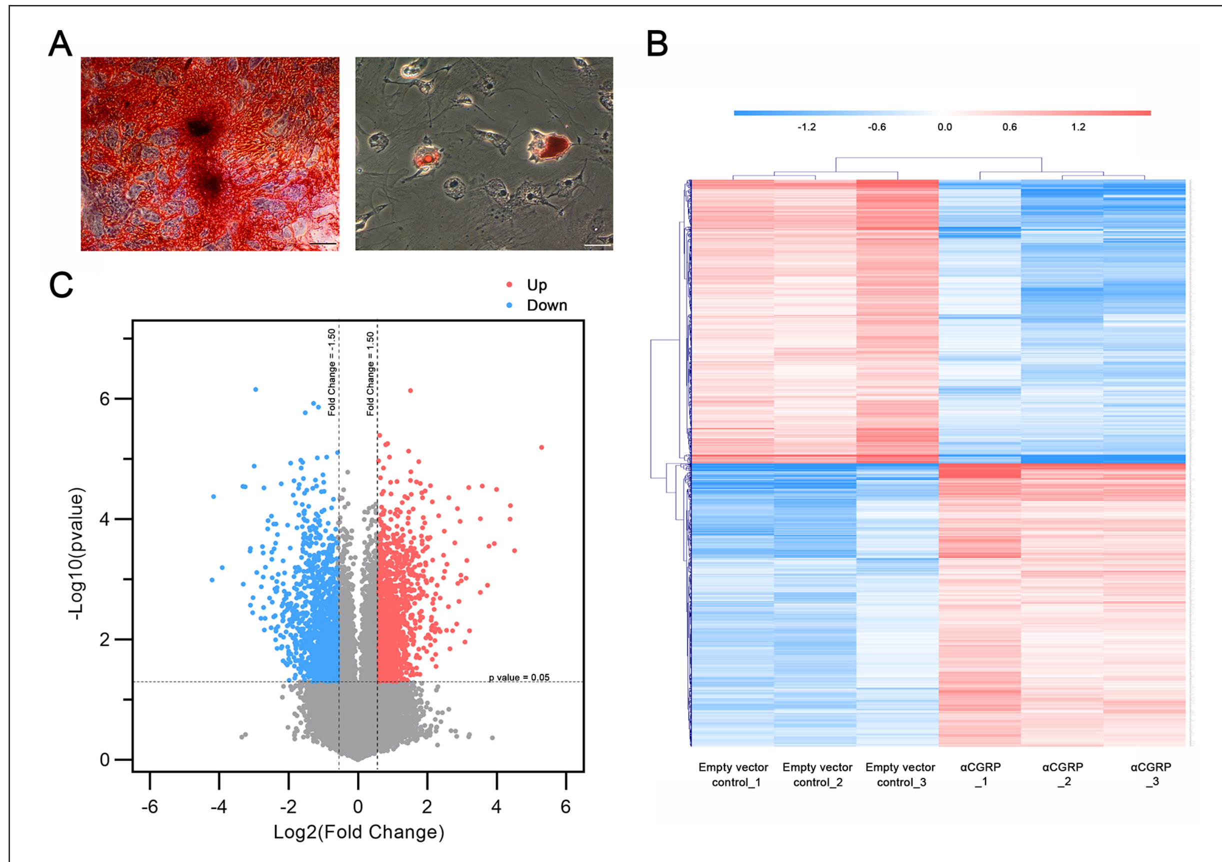

Figure 1. Identification of BMSCs and transcriptome analysis of αCGRP-mediated BMSCs using microarrays. (A) Mineralized matrix visualized following alizarin red staining of BMSCs after 21-day osteogenic induction. Scale bar=200μm. Oil Red O staining of BMSCs after 7-day adipogenic induction. Scale bar=40μm. (B) Hierarchical clustering analysis of differentially expressed genes. (C) Volcano plot of differentially expressed genes in empty vector control group vs αCGRP overexpression group. αCGRP: α-calcitonin-gene-related peptide; BMSCs: bone marrow mesenchymal stem cells.

The heatmap displayed that gene expressions in the same group were similar, but the transcriptional profiles in αCGRP overexpression group and empty vector control group were significantly distinct from each other (Fig. 1B). Then, we identified a set of genes (adjusted P value < 0.05, fold change (abs)>1.5) differentially expressed in these two groups (Fig. 1C).

Furthermore, we applied GO test to explore the roles and functions of all these differentially expressed genes and ranked them. Results in Fig. 2A indicated that αCGRP affected a series of biological processes in BMSCs. Among these, the upregulated GO functions were mainly related to immune system process, cell adhesion, positive regulation of gene expression, and so on. Those downregulated GO functions regulated by αCGRP were closely associated with mitotic nuclear division, cell cycle, DNA replication, and so on. On the other hand, pathway analysis of differently expressed genes was performed using KEGG database. Small P value implied that the expressed genes were highly enriched in this pathway. As shown in Fig. 2B, pathways enriched from upregulated genes in αCGRP lentivirus transfected BMSCs included cytokine-cytokine receptor interaction, MAPK signaling pathway, and so forth. Some important pathways enriched from genes with downregulated expression included DNA replication, cell cycle, mismatch repair, and so on. These data suggested that αCGRP could affect gene expressions, thus regulating biological behaviors of BMSCs.

MAPK Signaling Pathway Is Regulated by αCGRP in BMSCs

Based on the results of microarray assay, MAPK signal was selected to further study its role in regulating biological functions of αCGRP-mediated BMSCs. Belonging to a family of intracellular protein kinases, MAPK signaling cascades participated in a plenty of biological processes. ERK1/2 and p38, as the members of MAPK, were widely studied in previous research[22]. To further examine the effect of αCGRP on ERK1/2 and p38 MAPK pathways, αCGRP was added into BMSCs, and we monitored the activation of

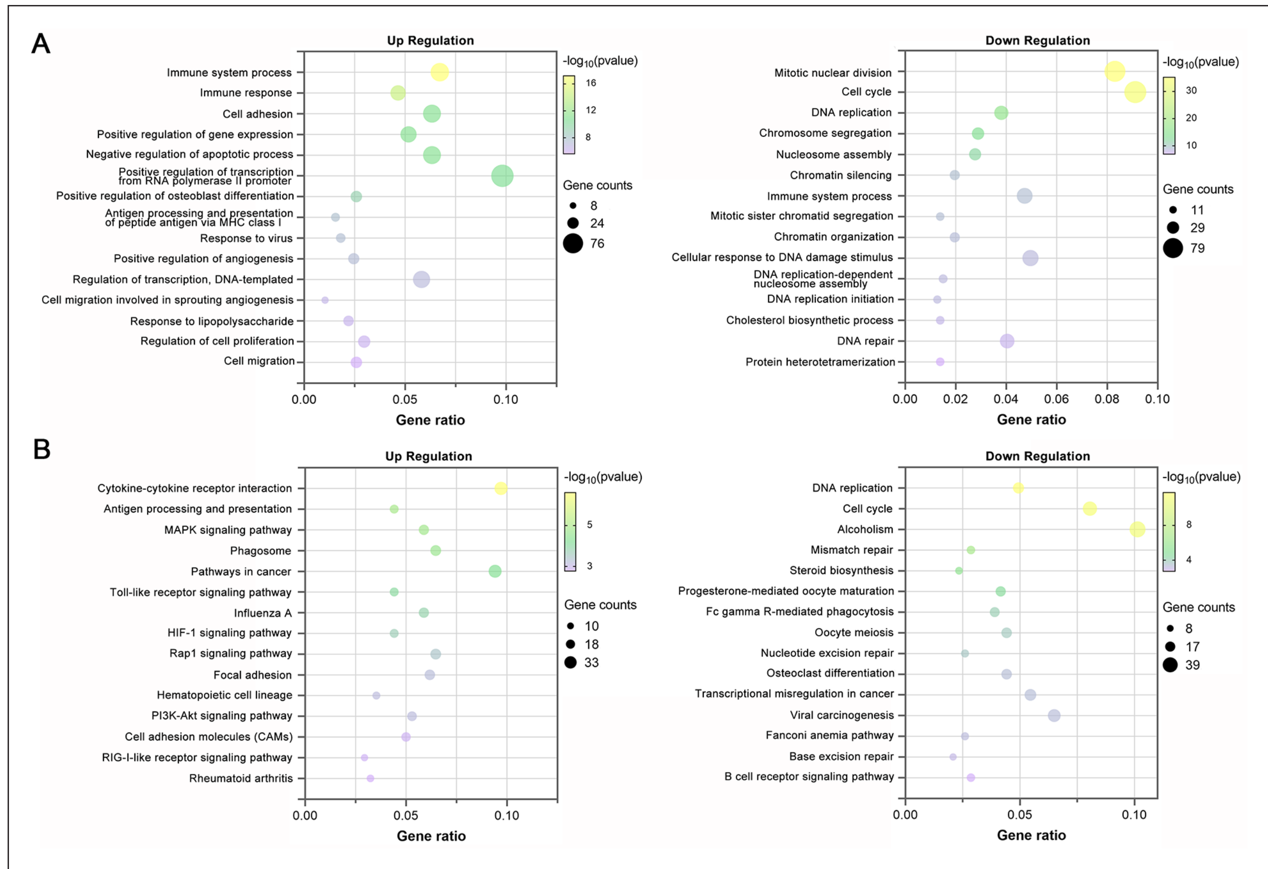

Figure 2. GO and KEGG analyses of αCGRP-mediated BMSCs. (A) The significant GO functions enriched by differentially expressed genes. (B) The enrichment of differentially expressed genes tested using KEGG pathway analysis. αCGRP: α-calcitonin-gene-related peptide; BMSCs: bone marrow mesenchymal stem cells; CAMs: cell adhesion molecules; DNA: deoxyribonucleic acid; GO: gene ontology; KEGG: kyoto encyclopedia of genes and genomes; MAPK: mitogen-activated protein kinase; MHC: major histocompatibility complex; HIF-1:hypoxia-inducible factor-1; RIG-I: etinoic acid-inducible gene-I.

these pathways at different time points. Results shown in Fig. 3A, B manifested that the phosphorylation of ERK1/2 and p38 was augmented along with prolonged time within 30 min after the addition of αCGRP. In other words, ERK1/2 phosphorylation was significantly enhanced during 15–30 min with the treatment of αCGRP, while p38 phosphorylation was drastically stimulated during 5–30 min. Of note, the phosphorylation of these two pathways reached peak about 30 min after the addition of αCGRP. Nevertheless, the expression level of ERK1/2 and p38 phosphorylation in BMSCs descended gradually after 30–60 min with αCGRP treatment. These observations illuminated that ERK1/2 and p38 MAPK signaling pathways in BMSCs could be upregulated by αCGRP and the activation processes were time-dependent. To further elucidate the role of αCGRP-MAPK signaling axis in BMSCs, cells were preincubated with U0126 (ERK1/2 pathway inhibitor) or SB203580 (p38 MAPK pathway inhibitor). In accordance with the results described above, the phosphorylation level of ERK1/2 and p38 was promoted in αCGRP group compared to control group. After U0126 or SB203580 was added, ERK1/2 and p38 phosphorylation was suppressed respectively (Fig. 3C, D).

αCGRP Regulates Biological Functions of BMSCs via MAPK Pathway

For cell viability, CCK-8 test revealed that αCGRP had negligible effects on the proliferative activity of BMSCs during 1–5 days. At 7 days, cell proliferation was upregulated by αCGRP. After inhibiting ERK1/2, cell viability was gradually suppressed as the incubation period prolonged, with notable differences at 7 days. When p38 was restrained by SB203580, BMSCs' proliferation significantly decreased compared to αCGRP group during 5–7 days (Fig. 4A). A decreased expression level of caspase-3, a key executioner in apoptosis, was detected in αCGRP group at 7 days, which was reversed by U0126 or SB203580 (Fig. 4B). Besides, ALP activity, ALP staining and alizarin red staining were conducted to evaluate the early and late differentiation potential of BMSCs after osteogenic induction. As displayed in Fig. 4C, D, we observed

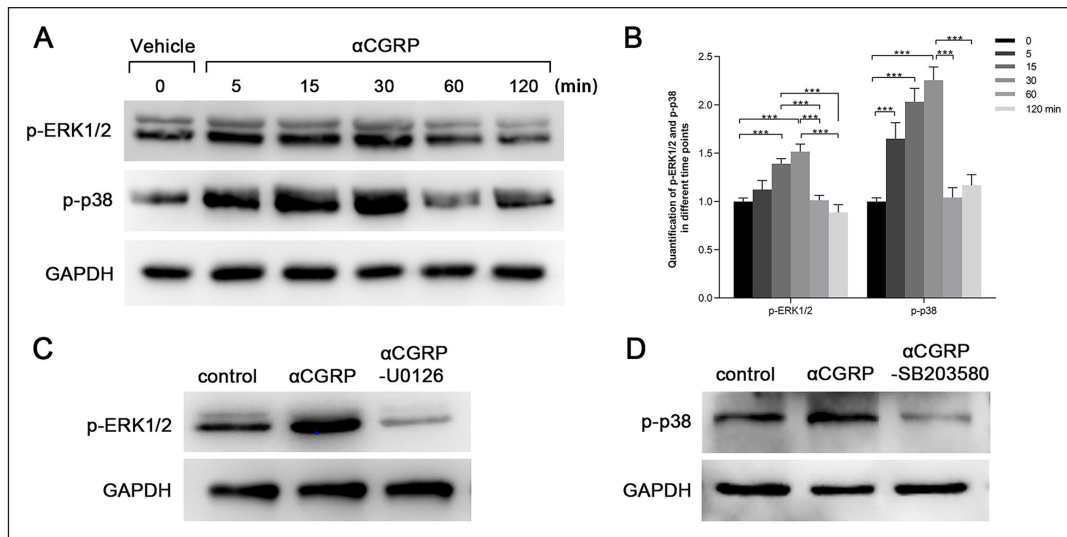

Figure 3. Effects of αCGRP on MAPK signaling pathway in BMSCs. (A, B) Qualitative and quantitative analyses of p-ERK1/2 and p-p38 expression levels in αCGRP-mediated BMSCs at different time points detected by western blot. GAPDH was used as loading control. (C, D) Protein levels of p-ERK1/2 and p-p38 in different groups. BMSCs were pretreated with or without αCGRP, with the addition of ERK1/2 inhibitor U0126 or p38 inhibitor SB203580. αCGRP: α-calcitonin-gene-related peptide; BMSCs: bone marrow mesenchymal stem cells; ERK1/2: extracellular signal-regulated kinases 1 and 2; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MAPK: mitogen-activated protein kinase; SD: standard deviation. Data obtained in 3 independent experiments are presented as mean ± standard deviation (SD), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

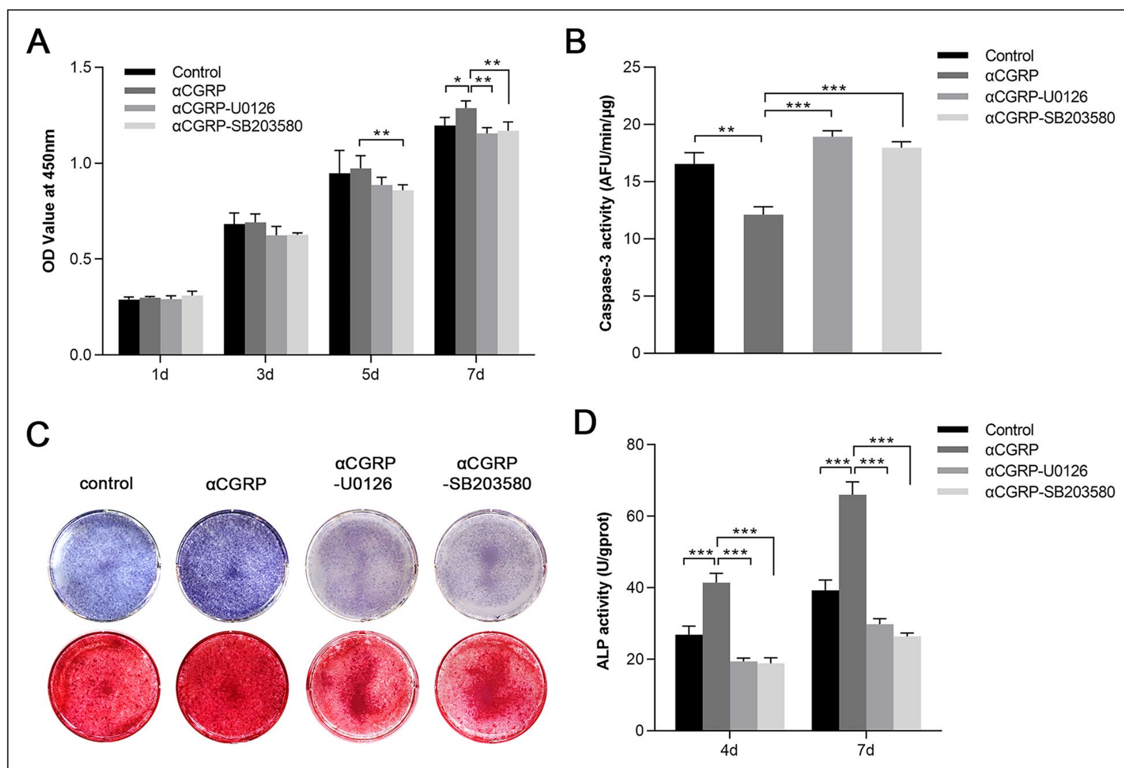

Figure 4. αCGRP effects on viability, apoptosis and differentiative capacity of BMSCs. (A) Cell proliferation determined by CCK-8 assay at 1d, 3d, 5d and 7d in BMSCs cultured in different conditions. (B) Caspase-3 activity after 7-day culture in different medium, AFU: arbitrary fluorescence units. (C) ALP and alizarin red staining of BMSCs after osteogenic induction in different groups. (D) ALP activity at 4d and 7d. αCGRP: α-calcitonin-gene-related peptide; ALP: alkaline phosphatase; BMSCs: bone marrow mesenchymal stem cells; CCK-8: Cell Counting Kit-8; OD: optical density. Values are expressed as mean ± standard deviation (SD) of 3 independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

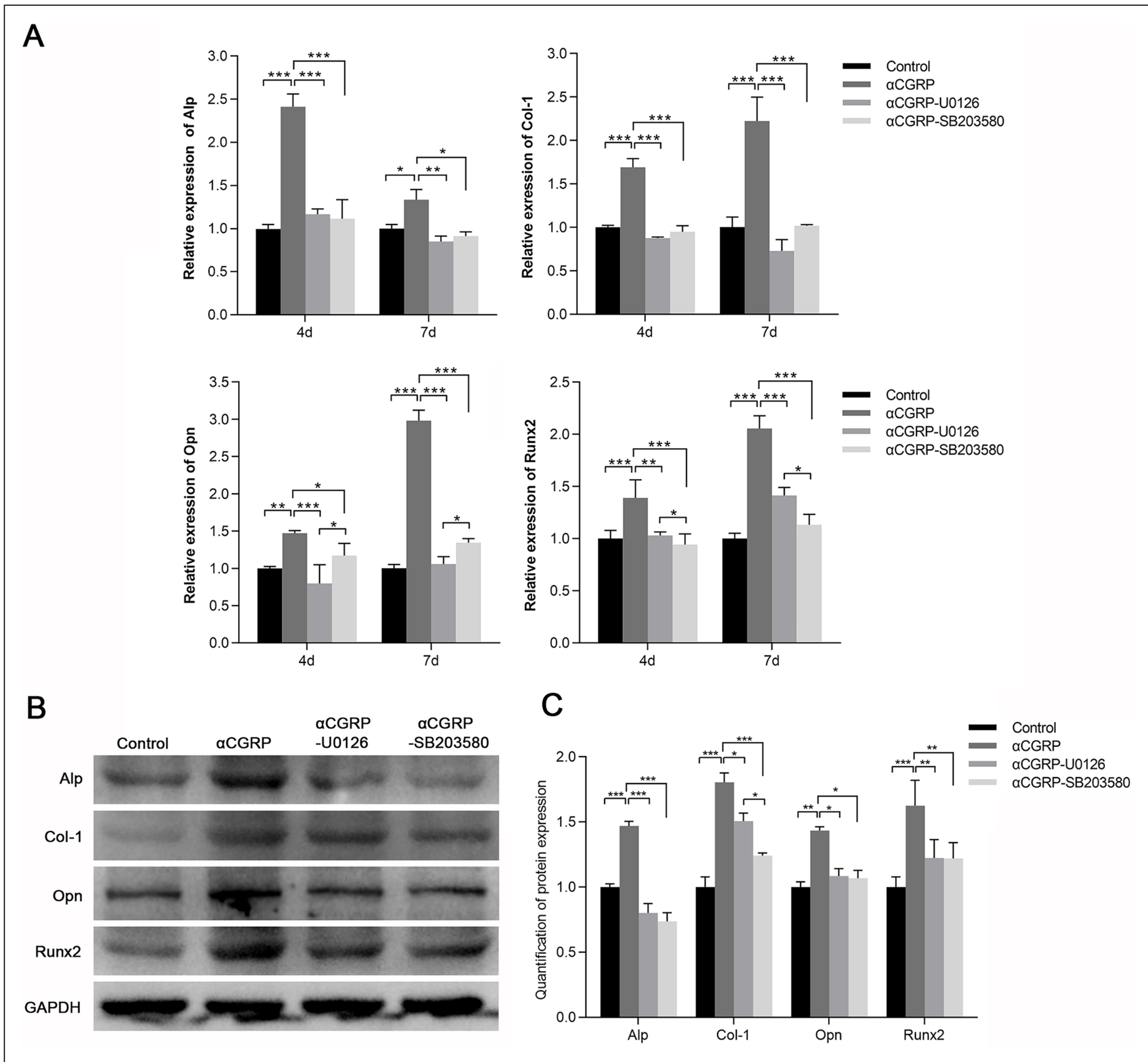

Figure 5. αCGRP influence on the expressions of osteogenic markers in BMSCs. (A) Relative expressions of Alp, Col-1, Opn and Runx2 tested by real-time qPCR. (B, C) Protein expression levels of Alp, Col-1, Opn and Runx2 were calculated using western blot analysis. αCGRP: α-calcitonin-gene-related peptide; Alp: alkaline phosphatase; BMSCs: bone marrow mesenchymal stem cells; Col-1: collagen type 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Opn: osteopontin; qPCR: quantitative polymerase chain reaction; Runx2: runt-related transcription factor 2. Values are expressed as mean ± standard deviation (SD) of 3 independent experiments, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

higher ALP activity and more calcium nodules in culture medium in αCGRP group than control group, which implied that αCGRP could potentiate differentiation and mineralization of BMSCs. However, inhibition of ERK1/2 and p38 led to impaired capacity of osteogenic differentiation, reflected in a lower level of ALP activity in BMSCs and decreased alizarin red-positive nodules.

Results in real-time qPCR demonstrated that the mRNA expressions of Alp, Col-1, Opn, and Runx2 were upregulated markedly in BMSCs with the treatment of αCGRP. But the addition of U0126 or SB203580 attenuated the rise in the levels of these osteogenic markers induced by αCGRP (Fig. 5A). Consistent with the data above, protein levels of those osteogenic factors showed similar variations according to western blot analysis (Fig. 5B, C). This finding suggested that αCGRP could regulate biological functions of BMSCs through MAPK signaling pathway (Fig. 6).

Discussion

As one of the neuropeptides identified in bone system, αCGRP is regarded as a bridge linking nervous system and

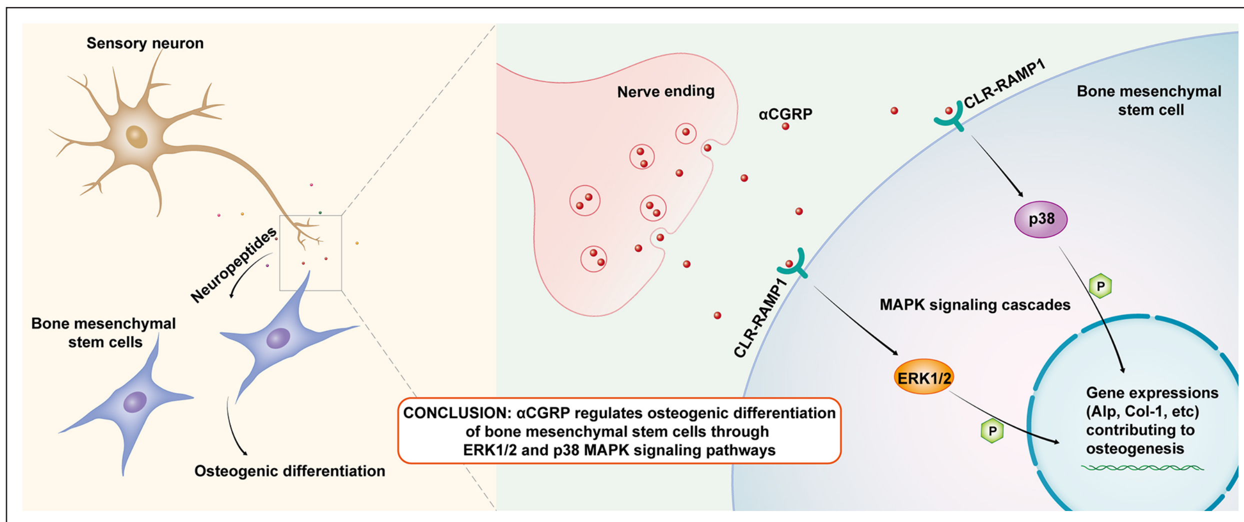

Figure 6. Graphical abstract of present study. αCGRP: α-calcitonin-gene-related peptide; Alp: alkaline phosphatase; Col-1: collagen type 1; CLR: calcitonin-like receptor; ERK1/2: extracellular signal-regulated kinases 1 and 2; MAPK: mitogen-activated protein kinase; RAMP1: receptor activity-modifying protein 1.

skeleton. Dense αCGRP-positive nerve fibers often coincide with the process of new bone formation, especially in the skeletal area undergoing reactive repair, hinting that αCGRP might be the essential prerequisite for bone turnover and remodeling[23]. And αCGRP participated in the complicated communications within bone-related cells including osteoblasts, osteoclasts, and BMSCs[18,24], thus positively maintaining bone homeostasis. To uncover the intrinsic mechanism of αCGRP in its osteogenic ability, microarray analysis was carried out in BMSCs treated with or without αCGRP overexpression. Enriched from differentially expressed genes, GO tests revealed that some upregulated biological processes in αCGRP overexpression group might be associated with osteogenic differentiation, such as cell adhesion, positive regulation of gene expression, positive regulation of transcription, and osteoblast differentiation. Some downregulated GO functions, including cell cycle, mitotic nuclear division, DNA replication initiation, were also likely to be related to osteogenic abilities of BMSCs[25]. These data demonstrated that αCGRP could modulate gene expressions, thus affecting biological behaviors of BMSCs, which expanded our understanding about the osteogenic roles of αCGRP in genetic and molecular aspects. Through KEGG analysis, we then screened potential signaling pathways implicated in the osteogenic mechanism in αCGRP-mediated BMSCs. Among those predominant pathways, MAPK signaling transduction came into notice.

MAPK cascade pathway is involved in a wide variety of important events such as cell survival, proliferation, differentiation, and so on[26]. As important members, ERK1/2 and p38 have been regarded as positive regulators of osteogenic differentiation and relative functions of bone-related cells, thus accelerating bone formation[27–29]. But other research has provided support for the negative effects of these subgroups of MAPK, indicating that their activation is responsible for osteoclast differentiation and maturation and might be the main cause for bone loss[30,31]. These controversial findings suggested that each MAPK cascade could have a unique specialty. And the biological properties might be conversed in different MAPK pathways in terms of extracellular stimuli, cell types, and microenvironment. On the basis of microarray analysis showing the differentially genes in αCGRP-induced BMSCs highly enriched in MAPK signaling, we therefore tried to find out the specific role of it in the αCGRP-mediated BMSCs. Given that the activity of MAPK depends on the phosphorylation at a tripeptide motif (threonine-x-tyrosine), which then acts on the downstream targets in sequence to modulate a series of physical processes[22,32], we investigated the effect of αCGRP on ERK1/2 and p38 phosphorylation in BMSCs. Both qualitative and quantitative approaches of western blot indicated that αCGRP stimulated ERK1/2 and p38 phosphorylation in a time-dependent manner, peaking at about 30 min after αCGRP addition. This might be attributed to the short activation life-time of MAPK module. Once its regulatory effects were exerted after the period of stimulation, the activation loop on amino acid residues would be dephosphorylated, and then MAPK signal terminated[33].

Previous research has indicated that αCGRP plays a critical role in the skeletal development and bone metabolism. The absence of αCGRP could lead to low bone mass/bone formation rate *in vivo*[16]. Besides, CGRP is able to stimulate the expression of bone morphogenic protein-2 (BMP-2) and osteoblast differentiation of human osteosarcoma-derived immature osteoblastic MG63 cells[34]. In pathological environment, CGRP could induce the differentiation of BMSCs derived from osteoporotic rat after long-term cell culture[35]. Consistently, our results revealed that αCGRP had the ability of improving osteogenic differentiation and mineralization

of BMSCs under physiological conditions, thus having positive influences on osteogenesis. The differences and novelty compared to the above studies are that we focus on the specific role of αCGRP in modulating primary mouse BMSCs' biological functions *in vitro* and mainly discuss the MAPK signal pathway involved. Importantly, we conducted microarray analysis to explore the alteration of gene expressions and signal transduction in this process, which could offer more proof to understand the intrinsic mechanism of αCGRP in bone formation. Furthermore, to verify whether αCGRP affected BMSCs through ERK1/2 and p38 MAPK signaling pathways, cells were pretreated with ERK1/2 or p38 MAPK-specific inhibitors—U0126 or SB203580. Our data showed that U0126 or SB203580 addition reversed the favorable effects of αCGRP, suggesting that ERK1/2 and p38 MAPK were important pathways in charge of the boosting effect of αCGRP on the differentiative capacity of BMSCs. On the other hand, several lines of evidence suggest that MAPK pathway is one of the most significant regulators for cell viability and apoptosis[36]. In this work, we found that αCGRP could enhance BMSCs' proliferation and inhibit apoptotic process. Inactivation of ERK1/2 or p38 MAPK abrogated these beneficial influences of αCGRP. These findings strengthened the significance of ERK1/2 and p38 MAPK signaling cascades in the αCGRP regulation in osteogenic properties of BMSCs. And these two members of MAPK might be intertwined to elicit the physical responses.

Of note, there were other biological processes displayed in GO analysis affected by αCGRP, for example, immune response regulation. It is described that αCGRP could affect immunological reactions in immune cells and some bone-related cells, indicating that αCGRP-mediated neuronal signaling might be a crucial regulator in the neuro-immuno-skeletal system[37,38]. Since αCGRP is known for an angiogenic promoter[39], upregulated regulation of angiogenesis shown in the results from microarray tests is also noteworthy. Related evidence in the literature has demonstrated that endothelial differentiation of BMSCs could be induced by αCGRP, which might be responsible for the promoted osteogenesis[40]. Further studies are required to tap more potentials and mechanisms of αCGRP in skeletal development. Importantly, we should pay attention to the crosstalk between BMSCs and other cell types since the microenvironment in nature is complicated and dynamic.

Conclusions

To summarize, our study elucidated that αCGRP could enhance osteogenic differentiation, proliferation and inhibit apoptosis of BMSCs. ERK1/2 and p38 MAPK signaling pathways, at least in part, contributed to the favorable role of αCGRP in this process. These findings shed light on the fact that αCGRP was an important regulator capable to coordinate the complex nerve-bone network, which might offer us a promising new strategy for the bone repair and regeneration.

Ethical Approval

This study was approved by the Ethics Committee of West China Hospital of Stomatology, Sichuan University (No.WCHSIRB-D-2017-162).

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with approved protocols of the Ethics Committee of West China Hospital of Stomatology, Sichuan University.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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